β-1,3-Glucanase Activity in Peanut Seed (*Arachis hypogaea*) is Induced by Inoculation with *Aspergillus flavus* and Copurifies with a Conglutin-Like Protein

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ABSTRACT

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Infection of peanut (*Arachis hypogaea*) seed by *Aspergillus flavus* and *A. parasiticus* is a serious problem that can result in aflatoxin contamination in the seed. Breeding resistant cultivars would be an effective approach to reduce aflatoxin accumulation. The objective of this study was to investigate the expression of the pathogenesis-related (PR) protein β-1,3-glucanase and the isoform patterns in peanut seed inoculated with *A. flavus*. Peanut genotypes GT-YY9 and GT-YY20 (both resistant to *A. flavus* infection) and Georgia Green and A100 (both susceptible to *A. flavus* infection) were used in this study. The activities of β-1,3-glucanase were similar in the uninfected seed of all genotypes, but increased significantly in the resistant genotypes after inoculation in comparison with the susceptible genotypes. An in-gel (native polyacrylamide gel electrophoresis [PAGE]) enzymatic activity assay of β-1,3-glucanase revealed that there were more protein bands corresponding to β-1,3-glucanase isoforms in the infected seed of resistant genotypes than in the infected seed of susceptible genotypes. Both acidic and basic β-1,3-glucanase isoforms were detected in the isoelectric focusing gels. Thin-layer chromatography analysis of the hydrolytic products from the reaction mixtures of the substrate with the total protein extract or individual band of native PAGE revealed the presence of enzymatic hydrolytic oligomer products. The individual bands corresponding to the bands of β-1,3-glucanase isoforms Glu 1 to 5 were separated on the sodium dodecyl sulfate-PAGE, resulting in two bands of 10 and 13 kDa, respectively. The sequences of fragments of the 13-kDa major protein band showed a high degree of homology to conglutin, a storage protein in peanut seed. Conglutin is reported as a peanut allergen, *Ara* h2. Our data provide the first evidences for peanut having β-1,3-glucanase activities and the association with the resistance to *A. flavus* colonization in peanut seed. We have not directly demonstrated that conglutin has β-1,3-glucanase activity.

Infection of peanut (*Arachis hypogaea* L.) seed by *Aspergillus flavus* Link:Fr. and *A. parasiticus* Speare is a serious problem. This infection can result in the contamination of the seed with aflatoxins, which are toxic fungal metabolites. These fungi are ubiquitous, being found virtually everywhere in the world. They are soilborne, but prefer to grow on high-nutrient media (e.g., seed). Progress has been made in an attempt to prevent aflatoxin contamination in crops (35) through crop management and handling, microbial ecology and biocompetitive microbes, and crop resistance through genetic engineering and conventional breeding (11,14).

In many plant–pathogen interactions, inducible pathogenesis-related (PR) proteins have been well documented (26). However, in peanut, very little has been done in the area of characterization of PR proteins and disease resistance. It has been reported that peanut contains endogenous chitinases (20), although there are no reports of endogenous β-1,3-glucanase in peanut. In corn, PR proteins include hydrolases (chitinases and β-1,3-glucanases), which degrade structural polysaccharides of the fungal cell wall (17); ribosome-inactivating-proteins (RIPs), which modify and inactivate foreign ribosomes (10,41); and zeamatin, which increases permeability of fungal cell membranes (10,36). β-1,3-Glucanase is a well-known PR protein that is constitutively expressed at low levels in plants and can be dramatically induced when plants are infected by fungal, bacterial, or viral pathogens (24). Several experiments have demonstrated that β-1,3-glucanase was partially able to degrade the cell wall and inhibit mycelial growth or spore germination of certain pathogenic fungi (24). In corn seed, β-1,3-glucanase has been proposed as a mechanisms for inhibiting the growth of *A. flavus* (27,32).

The development of host-plant resistance would be an effective approach to eliminate aflatoxin accumulation in peanut (11,15, 30). More understanding of host resistance mechanisms should speed the development of resistant cultivars. In the past decade, studies have identified some peanut germ plasm with resistance to *A. flavus* infection and aflatoxin production (2,15,16,25). Our laboratory and field studies have shown that some peanut germ plasm from China and India has lower *Aspergillus* fungal colonization in the laboratory and lower aflatoxin contamination in the field (12).

The overall goal of this research was to study the resistance mechanisms of peanut germ plasm and attempt to develop DNA markers that can be used in marker-assisted breeding to transfer the resistance to commercial cultivars. The specific objective of this study was to investigate the presence of PR protein β-1,3-glucanase in peanut and the isoform patterns in different peanut germ plasm inoculated with *A. flavus*.

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MATERIALS AND METHODS

Peanut genotypes. Four peanut genotypes were used in this study. GT-YY9 and GT-YY20 are resistant to *A. flavus* infection (25) and 'Georgia Green' and A100 are susceptible. All seeds were free of fungicides, insecticides, and other seed treatment materials, and were stored at 4°C until use.

Fungal strain and spore inoculum. Strain AF13, an aflatoxigenic strain of *A. flavus,* was used (13). The fungus was grown at 30°C in the dark on 10% V8 juice and 2% agar medium. Inoculum (10^6 conidia/ml) was obtained from 7-day-old culture and suspended in sterile deionized water containing Tween 20.

Inoculation. Intact, sound, and mature seed from each genotype were surface sterilized by immersion in 0.75% NaOCl for 5 min, rinsed in three changes of sterile distilled water, and soaked in sterile deionized water for an additional 45 min at room temperature. Then, these seed were rehydrated to 25% water content. Seed were immersed in a suspension of *A. flavus* inoculum, removed, and placed in cells (35 mm in diameter, 20 mm high) of a six-cell culture dish. Four dishes were sealed in a plastic food container (180 by 150 by 90 mm) and incubated at 30°C for 7 days under 100% relative humidity. Each treatment was replicated six times. The experiment was repeated twice.

Protein extraction. The infected and control seed (water-treated) were harvested at various time intervals after inoculation with *A. flavus* or a water control. After freezing in liquid nitrogen, the harvested seed were ground to a fine powder with a mortar and pestle and homogenized in a cold, 0.1 M sodium acetate buffer (pH 5.1) containing 4 M ascorbic acid, 2 mM 2-mercaptoethanol, and 2% polyvinyl-pyrrolidone (PVP). The crude extracts were centrifuged twice at 20,000 × *g* for 20 min at 4°C. The supernatants were collected, and protein concentration was determined by the method of Bradford (3). The proteins were precipitated from crude extracts with ice-cold acetone (1:5, vol/vol) overnight at –20°C. The precipitates were collected by centrifugation, washed at least twice with ice-cold 80% acetone, and air dried. The protein pellets were dissolved in distilled water. The protein solutions were stored at –20°C until they were analyzed for enzymatic activity assay and electrophoresis. The same procedure was used to extract proteins from mycelia of *A. flavus*.

Enzymatic activity assay of β-1,3-glucanase. β-1,3-Glucanase activity in crude extracts from different treatments was measured by colorimetric assay as described by Hwang et al. (18). This assay measures the rate of production of reducing sugars using laminarin (Sigma-Aldrich, St. Louis) as substrate. The β-1,3-glucanase activity was expressed in nkatal per milligram of protein. One nkatal was defined as the enzymatic activity catalyzing the formation of 1 nmol of glucose equivalent per second.

In-gel detection of β-1,3-glucanase and the isoforms. β-1,3-Glucanase was analyzed by polyacrylamide gel electrophoresis (PAGE) under native conditions using the Mini-PROTEIN II Dual Slab Cell System (Bio-Rad, Hercules, CA). Native-PAGE was performed on a mini-slab gel with a 15% separating gels and a 4% stacking gel. Isoelectric focusing (IEF)-gel electrophoresis was carried out on 7.5% polyacrylamide gels containing ampholyte (pH 3.0 to 10) (Sigma-Aldrich). The separation of each sample using native-PAGE and IEF was repeated three to five times.

Detection of β-1,3-glucanase isoforms after native PAGE and IEF was performed as described by Pan et al. (34). Gels were incubated in a solution containing 1% laminarin for 90 min at 40°C. β-1,3-Glucanase activity in the gels then was visualized by staining the gels for 10 min by boiling in a 1 M NaOH solution containing 0.3% (wt/vol) 2,3,5-triphenyl-tetrazolium chloride (Sigma-Aldrich). After staining, gels were placed in 7.5% acetic acid and stored at 4°C.

Thin-layer chromatography assay of hydrolytic products. The thin-layer chromatography (TLC) analysis of hydrolytic products by β-1,3-glucanase was conducted according to the method of Mohammaid and Karr (31). Protein extract and the individual band corresponding to each β-1,3-glucanase isoform (see partial purification and sequencing for detail) from the native PAGE from GT-YY20 seed 4 days after inoculation with *A. flavus* were used in TLC bioassay. The protein extract (20 μg) and the individual band were mixed with 5 μg of laminarin in 50 mM potassium acetate (pH 5.2) in total volume of 15 μl and incubated at 37°C for 90 min. The reaction mixture (2 μl) was spotted on Merck silica gel plate type 60 and dried with an air blower. The plates then were developed in a chamber containing 200 ml of solvent solution of 1-butanol/glacial acetic acid/water (55:30:15) until the front traveled two-thirds of the distance from the bottom. The silica plate was dried completely and subsequently developed for a second time in the same solvent solution. The air-dried plate was sprayed in a fume hood with a mixture of 50 ml of methanol containing 15 g of H_2SO_4 and 84.2 mg of ethylenediamine dihydrochloride (Sigma-Aldrich). It was heated at 120°C for 25 min until red spots appeared. The standard markers (2 μg) were glucose, fructose, and galactose as monosaccharides, and sucrose and trehalose as disaccharides. Controls were laminarin alone and laminarin plus boiled protein extract of 4-day-inoculated GT-YY20 seed.

Partial purification and in-gel sequencing. Four days after inoculation with *A. flavus*, a single electrophoretic separation of the protein extract of GT-YY20 was made on the native PAGE and the gel was stained with 0.3 M copper chloride for 5 min. All the bands that corresponded to β-1,3-glucanase activity were partially purified by excising from the gels, then labeled as isoform Glu 1 to 8, and destained three times in 20 ml of buffer (0.25 M EDTA and 0.25 M Tris) for 10 min with shaking. The destained bands of isoforms Glu 1 to 5 were equilibrated in sodium dodecyl sulfate (SDS)-PAGE sample buffer for 45 min, and then were laid and separated on SDS-PAGE (15% separating gel and a 4% stacking gel). After electrophoresis, the protein bands were excised and subjected to in-gel trypsin digestion and analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) to obtain the peptide sequence. A peptide sequence homology search against known proteins or translated open reading frames of expressed sequence tags (ESTs) in GenBank and SWISS-Prot were performed by BLAST (1).

RESULTS

The activities of β-1,3-glucanase in resistant and susceptible peanut genotypes were colorimetrically assayed in extracts from uninfected and infected seed (Fig. 1). The β-1,3-glucanase activity was detected and remained at a very low level in uninfected seed of both resistant and susceptible genotypes. In contrast, the activity was stimulated by infection with *A. flavus* in both resistant and susceptible genotypes. The β-1,3-glucanase activity in resistant genotypes increased remarkably 24 h after inoculation and reached its maximum level on the third day after inoculation (Fig. 1, upper panel). However, in the susceptible genotypes (Fig. 1, lower panel), β-1,3-glucanase activity increased gradually and slowly during the incubation and reached maximum levels on the fourth day after inoculation. The β-1,3-glucanase activity in resistant genotypes was three- to fourfold higher than that in susceptible genotypes.

In-gel assays were conducted in native PAGE to detect the isoform patterns of β-1,3-glucanase in resistant and susceptible genotypes and the differential expression of the isoforms as a result of infection of *A. flavus* (Fig. 2). Eight bands indicating different β-1,3-glucanase isoforms were detected in GT-YY20 and labeled as Glu 1 to 8 (Fig. 2). Band 8, Glu 8, was present in all samples, indicating constitutive expression of endogenous β-1,3-glucanases. Band Glu 7 might not be of peanut origin because this

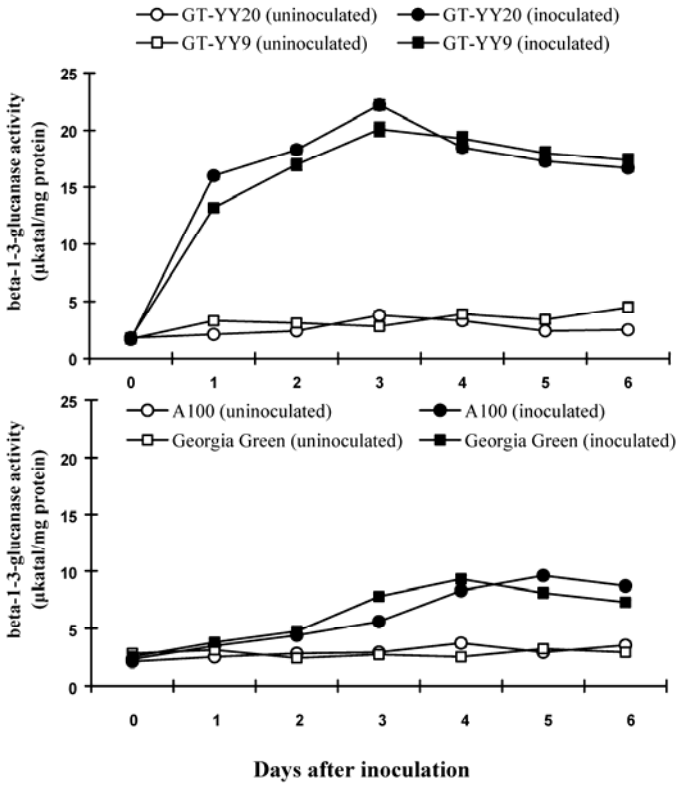

Fig. 1. Time curve of β-1,3-glucanase activity in seed extracts from resistance (GT-YY20 and GT-YY9) (upper panel) and susceptible (Georgia Green and A100) (lower panel) peanut genotypes inoculated with *Aspergillus flavus* and controls treated with water (uninoculated). Proteins were extracted from pooled samples of 50 seeds and each data point represents the average of three replicates ± SE.

band can be detected in protein extract of *A. flavus* (Fig. 2D, labeled as Af). Six isoform bands (designated as Glu 1, Glu 2, Glu 3, Glu 4, Glu 5, and Glu 6) on the gel seem specifically associated with *A. flavus* infection. The remarkable difference in response to *A. flavus* infection between the resistant and susceptible genotypes was the induction of new isoforms of β-1,3-glucanase. The resistant genotypes expressed more isoforms of β-1,3-glucanase and a quicker response to inoculation of *A. flavus* than did the susceptible genotypes. Three bands in GT-YY9 and five bands in GT-YY20 were observed 24 h after inoculation (Fig. 2C and D). No additional bands appeared in susceptible genotypes until 3 and 4 days after inoculation with *A. flavus* (Fig. 2A and B).

To determine the isoelectric points (pI) of the β-1,3-glucanase isoforms on IEF gels, crude protein extract from GT-YY20 at 4 days after inoculation were isoelectric focused on 7.5% IEF gel with a pH range of 3.0 to 10 (Fig. 3). Eight bands were detected on IEF gel, representing five acidic (pI 4.3, 5.1, 5.6, 6.5, and 6.9) and three basic (pI 7.7, 7.9, and 8.3) β-1,3-glucanase isoforms (Fig. 3).

TLC analysis of the hydrolytic products revealed the presence of different monomers and oligomers in the reaction mixtures of the substrate with protein extracts from GT-YY20 or the substrate with the individual bands corresponding to β-1,3-glucanase isoforms Glu 1 to 5 (Fig. 4). The control samples were laminarin alone or laminarin incubated with boiled protein extract. There were no hydrolytic products in the control (Fig. 4, lanes 6 and 7). This further indicated that both protein extracts and the corresponding bands have the enzymatic function of β-1,3-glucanase, even when the individual bands had lower hydrolytic activities (Fig. 4, lanes 11 to 15) due to lower concentrations. The intensity of β-1,3-glucanase activities was different among the isoforms Glu 1 to Glu 5.

The bands corresponding to β-1,3-glucanase isoforms (Glu 1 to Glu 6) were excised and subjected to SDS-PAGE. Interestingly,

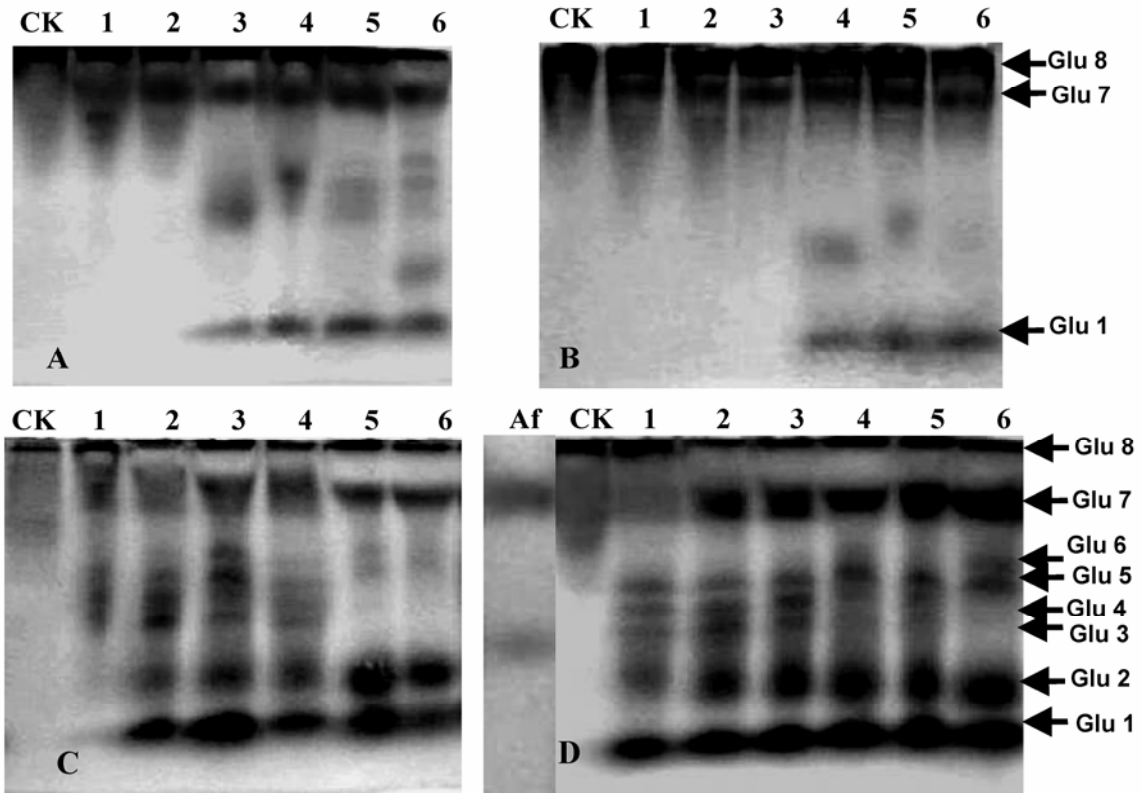

Fig. 2. Isoform patterns of β-1,3-glucanase activities on a native polyacrylamide gel (PAGE). Each lane was loaded with 150 µg of proteins extracted from peanut seed of **A,** A100, **B,** Georgia Green, **C,** GT-YY9, and **D,** GT-YY20 with infection of *Aspergillus flavus* after 1 to 6 days (lanes 1 to 6) and without infection (lane CK). Protein extract from *A. flavus* was loaded as a control (lane Af).

five bands, Glu 1 to Glu 5, resulted in two protein bands on SDS-PAGE with molecular masses of ≈13 and 10 kDa, respectively (Fig. 5). Glu 6 showed multiple bands on SDS-PAGE (data not shown). The 13-kDa major protein band was used for in-gel trypsin digestion and sequencing by ESI-MS/MS. Three internal peptides were sequenced as CCDELDQMENTER, ELMNLPQQCNFR, and QMVQQFK (Table 1). The sequences were submitted through a BLAST search and all three peptide sequences were 100% matched to the sequence of conglutin from peanut (33).

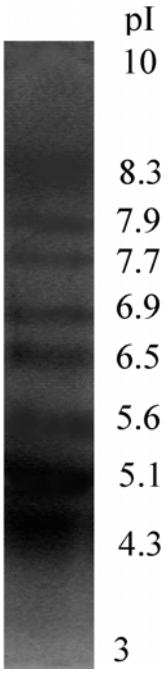

Fig. 3. Detection of β-1,3-glucanase isoforms on isoelectric focusing-gel electrophoresis. Crude total protein extract (150 μg) from GT-YY20 seed 4 days after inoculation with *Aspergillus flavus*. Isoelectric points (pI) of pH were determined as five acidic (pI 4.3, 5.1, 5.6, 6.5, and 6.9) and three basic (pI 7.7, 7.9, and 8.3) β-1,3-glucanase isoforms.

DISCUSSION

In this study, we presented evidence that peanut has β-1,3-glucanase PR proteins. The isoforms of β-1,3-glucanase were revealed on native PAGE differently in different genotypes as a result of infection of *A. flavus*. In the untreated control seed, the baselines of endogenous β-1,3-glucanase were similar in all tested genotypes. In the seed inoculated with *A. flavus*, the activities of β-1,3-glucanase were increased significantly in the resistant genotypes in comparison with the susceptible genotypes. Eight isoforms were detected in an in-gel (native PAGE) assay and labeled as Glu 1 to 8, consecutively. Although Glu 8 was expressed constitutively, Glu 1 to 6 were expressed in response to the infection of *A. flavus*, and Glu 7 may be produced by the fungus itself. These eight isoforms also were revealed on the IEF gel. TLC analyses of the hydrolytic products from the reaction mixtures demonstrated the presence of the oligomers, suggesting the pres-

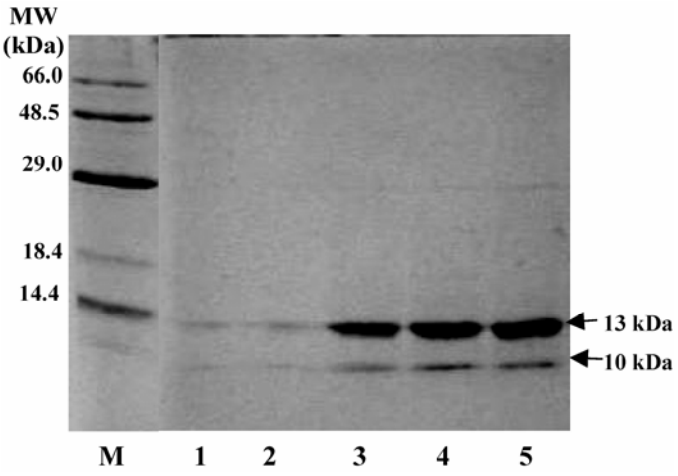

Fig. 5. Partially purified β-1,3-glucanases revealed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The bands corresponding to β-1,3-glucanase isoforms Glu 1 to 5 on native PAGE were excised and subsequently subjected to SDS-PAGE. Lanes are protein molecular weight markers (M), Glu 1 to 5 (lanes 1 to 5).

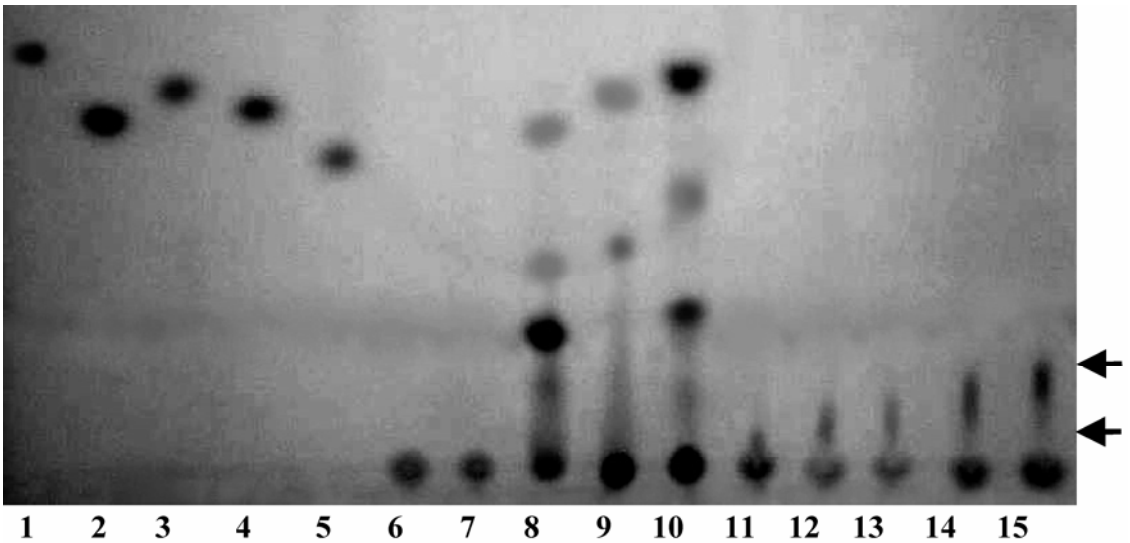

Fig. 4. Chromatogram of thin-layer chromatography silica gel plate of hydrolytic products generated as a result of β-1,3-glucanase-catalyzed hydrolysis of laminarin in the protein extracts of GT-YY20 seed infected with *Aspergillus flavus* and partially purified protein bands from native gels corresponding to β-1,3-glucanase isoforms. The standard markers were glucose (lane 1), fructose (lane 2), galactose (lane 3), sucrose (Lane 4), and trehalose (lane 5). The lanes loaded with different mixtures were the substrate laminarin alone (lane 6), laminarin plus boiled, 4-day-infected seed protein extract (lane 7), plus 2-day-infected seed extract (lane 8), 4-day-infected seed extract (lane 9), 6-day-infected seed extract (lane 10), and the substrate laminarin plus the individual bands from the native gels (Fig. 2) Glu 1 (lane 11), Glu 2 (lane 12), Glu 3 (lane 13), Glu 4 (lane 14), and Glu 5 (lane 15). Lanes 11 to 15 suggest that these individual bands have hydrolytic activities as the hydrolytic products generated (arrows).

TABLE 1. Amino acid sequence alignment of peanut conglutin and the sequences of the 13-kDa protein peptides which had β-1,3-glucanase activity

Conglutin	71 SSDQQQRCCDELDQMENTERCM---111 DRQMVQQFKRELMNLPQQCNFRAPQR 146	
13-kDa protein	CCDELDQMENTER	QMVQQFK ELMNLPQQCNFR

ence of the enzyme β-1,3-glucanase. The peptide sequences of the protein corresponding to the band of β-1,3-glucanase isoforms from native PAGE have homology to conglutin, a storage protein in peanut seed.

Most plants respond to pathogen attacks by synthesizing an assortment of PR proteins. Both chitinase and β-1,3-glucanase are well-studied PR proteins which result from plants responding against diseases (5,21,23,39). In corn, some studies have shown a positive relationship between β-1,3-glucanases activity and resistance against *A. flavus*, and some isoforms may be more responsible for defense against *A. flavus* (19,27). In comparison with the controls in this study, the increased activities of β-1,3-glucanase in both resistant and susceptible genotypes were evidenced after seed were challenged by *A. flavus*. The activities increased to much higher levels in the resistant genotypes than they did in the susceptible genotypes, indicating that the induction of β-1,3-glucanase could be a part of the general defense against *A. flavus* infection in peanut seed. Lozovaya et al. (27) reported that the elevated β-1,3-glucanase activities in corn kernels were correlated with the lower *A. flavus* infection in the resistant genotypes compared with the susceptible genotypes.

Ji et al. (19) reported the observation of β-1,3-glucanase isoforms in corn kernels that were induced by infection of *A. flavus*. In this study, we also observed the differences of isoform patterns between the resistant and susceptible peanut genotypes. The differences are the number of isoforms induced, the relative concentrations, and the time in response to fungal infection. Isoforms of Glu 1 to Glu 5 have hydrolytic activities as evidenced in TLC plates. Several oligosaccharides were observed as the results of laminarin hydrolysis by Glu 1 to Glu 5. These indicated that the isoforms of Glu 1 to Glu 5 may play both a direct protective role by degrading the *A. flavus* cell wall and an indirect role in defense response by releasing oligosaccharides from the fungal cell wall as elicitors. The elicitors might stimulate phytoalexin accumulation and production of PR proteins in seed (22,32, 37,38).

Quite interestingly, isoforms Glu 1 to Glu 5 were found containing two proteins, a minor 10-kDa and a major 13-kDa protein, in SDS-PAGE. The differences of the isoforms Glu 1 to Glu 5 in native-PAGE probably are related to their polarities. The polarity is determined by three-dimensional conformation of β-1,3-glucanase isoforms, which are revealed in different positions on the Native PAGE. The peptide sequences showed that the 13-kDa protein is highly homologous to conglutin of peanut. Conglutin, a peanut storage protein, was classified as the peanut allergen, *Ara* h2 (4), and its biological functions are still unclear. *Ara* h2 (conglutin) had been found to act as a trypsin inhibitor (28). A 75-fold increase in expression of conglutin gene was observed as peanut seed developed from seed maturity stage 1 to 2 (33). Very recently, conglutin expression also was found to be induced by drought stress and *A. flavus* infection in immature seed using peanut microarray analysis in our laboratory (M. Luo, E. Lee, and B. Z. Guo, *unpublished data*). In the present study, we demonstrated that the isoform patterns were induced by infection of *A. flavus* and the 13-kDa conglutin-like protein might function as a β-1,3-glucanases. Chenault et al. (6) noted in the assay of hydrolase activity in transgenic peanut that some degradation of substrate was observed during glucanase assays on nontransformed control peanuts. Our results suggest that this 13-kDa conglutin-like protein may have β-1,3-glucanase activity. To our best knowledge, this may be the first report that peanut has β-1,3-glucanases and that the β-1,3-glucanase isoforms may be associated with resistance to *A. flavus*.

The dual functions of some PR proteins have been documented in the literature. Some thaumatin-like proteins have been shown to possess β-1,3-glucanase activity (9). Sporamin (sweet potato storage protein) and patatin (potato storage protein) have been reported to act as a trypsin inhibitor and β-1,3-glucanases, respectively (40,42). Recently, ocatin, a storage protein in *Oxalis tuberosa*, was found to have antibacterial and antifungal functions (8), and a 32-kDa storage protein also was reported to possesses chitinase activity in *Medicago stativa* taproots (29). In peanut, Maleki et al. (28) reported that peanut allergy protein, *Ara* h2 (conglutin), had trypsin inhibitor activity. More recently, Dodo et al. (7) reported that peanut allergen *Ara* h3 also may function as a trypsin inhibitor. In this study, we have not directly demonstrated that peanut conglutin has β-1,3-glucanase activity. Further study will be needed to characterize the dual functions of this storage protein in peanut.

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